ORIGINAL ARTICLE

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Tumor mRNA–loaded dendritic cells elicit tumor-specific CD8+ cytotoxic T cells in patients with malignant glioma

Abstract In this study, we demonstrate that tumor mRNA–loaded dendritic cells can elicit a specific CD8+ cytotoxic T-lymphocyte (CTL) response against autologous tumor cells in patients with malignant glioma. CTLs from three patients expressed strong cytolytic activity against autologous glioma cells, did not lyse autologous lymphoblasts or EBV-transformed cell lines, and were variably cytotoxic against the NK-sensitive cell line K-562. Also, DCs-pulsed normal brain mRNA failed to induce cytolytic activity against autologous glioma cells, suggesting the lack of autoimmune response. Two patients' CD8+ T cells expressed a modest cytotoxicity against autologous glioma cells. CD8+ T cells isolated during these ineffective primings secreted large amounts of IL-10 and smaller amounts of IFN-γ as detected by ELISA. Type 2 bias in the CD8+ T-cell response accounts for the lack of cytotoxic effector function from these patients. Cytotoxicity against autologous glioma cells could be significantly inhibited by anti-HLA class I antibody. These data demonstrate that tumor mRNA–loaded DC can be an effective tool in inducing glioma-specific CD8+ CTLs able to kill autologous glioma cells in vitro. However, high levels of tumor-specific tolerance in some patients may account for a significant barrier to therapeutic vaccination. These results may have important implications for the treatment of malignant glioma patients with immunotherapy. DCs transfected with total tumor RNA may represent a method for inducing immune responses against the entire repertoire of glioma antigens.

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Introduction

Despite advances in radiation and chemotherapy along with surgical resectioning, the prognosis for patients with malignant glioma is poor. Among the new treatments currently being investigated for malignant glioma, immunotherapy is theoretically very attractive, since it offers the potential for high tumor-specific cytotoxicity [7, 18, 23]. Active immunotherapy, intended to induce antigen-specific T-cell responses to tumor antigens, has advanced as a concept for the treatment of glioma [2, 9, 10, 11, 12, 20, 23].

To date, there is limited presentation on the antigenic peptides and CTL epitopes presented by human tumors, with the exception of melanoma [5, 6]. However, several reports have shown that multiple epitopes can be recognized by T cells on human tumors [15, 21]. Therefore, an alternative strategy for effective vaccination of tumor patients may be the use of unfractionated tumor-derived materials such as whole tumor cells, peptides, or proteins isolated from tumor cells. In this regard, effective tumor immunity in several murine glioma models has been induced using professional antigen-presenting cells (APCs) such as dendritic cells (DCs) pulsed with unfractionated tumor-derived antigens in the form of peptides [12], cell lysates [2, 9], cDNA [20] or messenger RNA (mRNA) [10]. Vaccination of mice with DCs loaded with antigens results in tumor-specific cytotoxic T-lymphocyte (CTL) responses capable of rejecting implanted tumors.

Several groups have recently established the significant role played by DCs in the immune system and provided a rationale for using DCs as adjuvants for human immunotherapy [19, 22]. DCs are the most effective APCs at activating naïve T cells [19, 22], and recently the combination of GM-CSF and IL-4 has been

shown to generate large numbers of DCs from peripheral blood monocyte precursors [17].

It is reported that murine and human DCs transfected with mRNA can stimulate CTL responses in vitro and in vivo [4, 13], and treatment of tumor-bearing mice with DCs transfected with tumor RNA led to a significant reduction in metastasis [4] and improved survival rates [3]. The advantage of transfecting DCs with RNA-encoding antigens is that mRNAs encode multiple epitopes that can bind to many HLA alleles. RNA-transfected DCs may be used to stimulate T-cell responses in patients without prior knowledge of their HLA haplotype.

The purpose of this study is to evaluate the hypothesis that DCs loaded with the total RNA of autologous glioma cells could stimulate a CTL response capable of lysing the autologous glioma cells in vitro. Here, we report the in vitro induction of HLA class I–restricted $CD8^+$ CTLs in patients with malignant glioma. These results provide evidence that T-cell responses specific for undefined tumor antigens can be generated in patients with glioma with total tumor RNA–transfected DCs. These results may have important implications for treatment of malignant glioma with active or adoptive immunotherapy.

Materials and methods

Patients

Five patients who had undergone subtotal removal for malignant glioma provided tumor tissue and peripheral blood mononuclear cells (PBMCs). Specimens were obtained at the time of surgery through the Neurosurgical Department at the Niigata University under approval of the Institutional Review Board. All patients had glioblastoma according to the World Health Organization (WHO) criteria. Median age was 62, and median Karnofsky perfomance status (KPS) was 82 (Table 1). Patients did not receive any form of therapy prior to surgery. The normal brain was obtained from lobectomy at benign tumor surgery of 64-year-old women.

Tumor cell lines

The natural-killer (NK) sensitive target K562 was purchased from Riken Cell Bank (Tsukuba, Ibaragi) and was maintained at 37°C with 5% CO_2 in RPMI 1640 (Invitrogen, Tokyo), supplemented with 10% fetal bovine serum (FBS; Invitrogen). Fresh autologous tumor samples were obtained from surgical specimens from all patients and were reduced to single cell suspensions under sterile conditions at room temperature. Tumor tissue was mechanically disrupted and propagated in Matrigel basement membrane matrix (Becton Dickinson, Bedford, MA) in RPMI 1640 supplemented

Table 1 Patient characteristics

Case	Age	Sex	Pathological diagnosis	KPS
1	53	F	Glioblastoma	80
2	69	M	Glioblastoma	90
3	60	M	Glioblastoma	70
4	62	M	Glioblastoma	80
5	20	F	Glioblastoma	90

with 10% FBS, 2-mmol/l L-glutamine, 100-μg/ml streptomycin, and 100-U/mL penicillin. Fresh tumor cell lines were maintained initially in RPMI 1640, supplemented with 10% FBS at 37°C with 5% CO_2. The cell suspension was then washed three times in RPMI and thereafter seeded in 75-cm^2 tissue culture flasks (Corning, Cambridge, MA).

Tumor and GFP mRNA preparation

About 10^7 autologous tumor cells cultured in RPMI 1640 supplemented with 10% FBS were washed three times with phosphate-buffered saline (PBS) pH 7.4 and harvested by scraping. Total RNA was isolated from the autologous tumor cells of each patient using RNeasy RNA isolation Kit (Qiagen, Tokyo) according to manufacturer's protocol. The amount of total RNA was about 100 μg, and the quality was evaluated by gel electrophoresis. The ratio of 28S/18S was over 1.8 in all cases.

The genes for green fluorescent protein (GFP) were amplified by reverse transcription polymerase chain reaction (RT-PCR) from pEGFP-N1 (Clontech Laboratories, Palo Alto, CA) and cloned into an RNA-transcription plasmid containing T7 and CMV promoters. The vector was polyadenylated with an adenines downstream of the insert to add a poly (A) tail to the in vitro–transcribed RNA. The plasmid DNA was then in vitro–transcribed using a commercial kit (Message, Machine, Ambion, Austin, TX). The production of full-length capped RNA was confirmed by gel electrophoresis.

Dendritic cells generation and pulsing by tumor mRNA

A leukapheresis was performed on the patients and PBMCs were isolated using Ficoll-Hypaque density gradient centrifugation. DCs were generated from PBMCs in serum-free AIM-V medium (Invitrogen). Briefly, PBMCs were placed into 6-well culture plates (Costar) in AIM-V medium at 1×10^7/3 ml per well. After 4 h at 37°C, nonadherent cells were removed, and the adherent cells were cultured at 37°C in a humidified 5% CO_2 incubator, in medium supplemented with human recombinant GM-CSF (rGM-CSF) (1,000 U/ml; Immunex, Seattle, WA) and IL-4 (500 U/ml; Genzyme, Cambridge, MA). After 7 days of culture, DCs were harvested for pulsing with tumor mRNA as described below. The DC purity (i.e., cells strongly expressing HLA-DR$^+$, CD80$^+$, and CD86$^+$) ranged from 75 to 90% of the total cell population. No significant differences were noted in the expression of HLA-DR or costimulatory molecules (i.e., CD80 and CD86) among DC cultures derived from these five patients (data not shown).

DCs (5×10^6 cells/ml) in AIM-V medium were transfected with autologous tumor RNA. DCs to be used were transfected with RNA in the presence of lipid. RNA in 250-μl Opti-MEM (Invitrogen) and the cationic lipid DOTAP (Boehringer Mannheim, Indianapolis, IN) in 250-μl Opti-MEM, were mixed in 12×75-mm polystyrene tubes (Falcon, Oxnard, CA) at room temperature for 10 min. The amount of total RNA used was 10 μg per 10^6 DCs. The RNA to lipid ratio was 1:3. The complex was added to DCs (5×10^6 cells/ml) in Opti-MEM and incubated at 37°C for 1 h. DCs were washed and cultured overnight at 37°C in the presence of GM-CSF and IL-4 before use.

In vitro induction of tumor-specific CTL responses

Autologous PBMCs obtained were cocultured with antigen-pulsed DCs in the presence of IL-2 and IL-7 (both 10 U/ml; Genzyme). After 14 days, CD8$^+$ T cells were isolated using CD8 microbeads (Miltenyi Biotech, Sunnyvale, CA) according to the manufacturer's protocol. The purity of CD8$^+$ cells was 95% or more by FACS analysis (data not shown). The CD8$^+$ T cells were cultured in RPMI 1640 supplemented with 10% FBS and 20 U/ml of IL-2 at

37°C. Two days after purification, T cells were re-stimulated with antigen-pulsed DCs. CTL assays were done 7 days after re-stimulation.

Specific cytotoxic activity was determined using the formula % specific release = ([experimental release − spontaneous release]/[total release − spontaneous release]) × 100. Spontaneous release was less than 10% of total release. Standard errors of the means of triplicate cultures were less than 5%.

As negative control targets, autologous lymphoblasts were prepared by 3-day stimulation with Con-A (Invitrogen; 1 g/ml) in RPMI 1640 plus rIL-2 (25 U/ml), while EBV-transformed autologous lymphoblastoid B-cell lines (LCL) were established by co-culture of PBMCs with EBV-containing supernatant from the B95.8 cell line (Riken Cell Bank) in the presence of 1-g/ml cyclosporin A (Sandoz, Camberley, UK) and were maintained in RPMI supplemented with 10% human AB serum.

Cytotoxic activity

A 6-h chromium (^{51}Cr) release assay was performed as previously described [20] to measure the cytotoxic reactivity of DC-tumor mRNA stimulated CD8$^+$ T lymphocytes. In addition to autologous tumor cells, K562 cells were used as a target for the detection of NK-cell activity. Con-A activated peripheral blood lymphocytes and/or EBV-transformed LCL were used as autologous control targets. To determine the structures on the effector and target cells involved in lysis, monoclonal antibodies (MAbs) were used to block cytotoxicity. ^{51}Cr-labeled tumor targets were preincubated with MAbs specific for HLA class I (W6/32; Dako, Carpinteria, CA) and its isotype control (IgG$_1$k MAb isotype standard; PharMingen). The effector cells and ^{51}Cr-labeled targets were then incubated in a final volume of 200 μl per microwell at 37°C with 5% CO$_2$. Cytotoxicity assays were also evaluated with purified CD8$^+$ T cells 3 weeks after stimulation of T-cell cocultures with normal brain mRNA–pulsed DC as described above.

Enzyme-linked immunosorbent assay

Two-milliliter CD8$^+$ T lymphocyte suspensions at a concentration of 1.0×10^6/ml in RPMI-complete medium supplemented with 5% FBS were plated into 6-well plates and were cultured at 37°C with 5% CO$_2$. These cells were unstimulated or stimulated by OKT3 (CLB, Amsterdam, The Netherlands), final concentration 125 ng/ml, to stimulate cytokine production. After 48 h of incubation, the supernatants were sampled and frozen at −80°C until use. Cytokine levels in the supernatant were determined by enzyme-linked immunosorbent assay kits (Quantikine, R&D Systems,

Minneapolis, IN) according to manufacturer's instructions. Sensitivity of the assay for IL-10 and IFN-γ was 1.5 and 0.3 pg/ml, respectively. Samples and standards were tested in triplicate.

Phenotypic analysis of T cells and tumor cells

Enriched cultures of CD8$^+$ T cells were phenotyped. Flow cytometry was performed using MAbs directly conjugated against the following human leukocyte antigens: FITC-anti-CD3, CD4, CD8, CD56, CD16, CD25, HLA-DR (all Becton Dickinson, San Jose, CA) and analyzed on a FACScan (Becton Dickinson). Autologous tumor cells were analyzed for MHC class I expression. Briefly, tumor cells were harvested with 0.25% Trypsin in RPMI and washed three times in PBS containing 2% FBS. Isotype-matched controls (FITC-anti-IgG$_2$a) from Becton-Dickinson was also used.

Results

HLA class I expression by tumor cells

MHC class I expression was investigated by FACS analysis on the tumor cell lines established from all five patients. As shown in Fig. 1, all tumor cell lines expressed MHC class I molecules.

Relative transfection efficiency

DCs were transfected with RNA for green fluorescent protein (GFP) using the above transfection techniques and incubated for 24 h, allowing for RNA translation into intracellular GFP. Transfected DCs were then analyzed by flow cytometry for the presence of GFP. Transfection of GFP mRNA resulted in the transfection efficiency of 22 ± 6%.

Tumor-specific CD8$^+$ cytotoxic T-cell responses

Cytotoxicity assays were evaluated with purified CD8$^+$ T cells 3 weeks after stimulation of T-cell cocultures

Fig. 1 Expression of MHC class I molecules on glioma cells. Surface antigen expression was measured by FACS as described in "Materials and methods." A representative experiment is shown. *Open profile* isotype control, *solid profile* anti-MHC class I MAbs
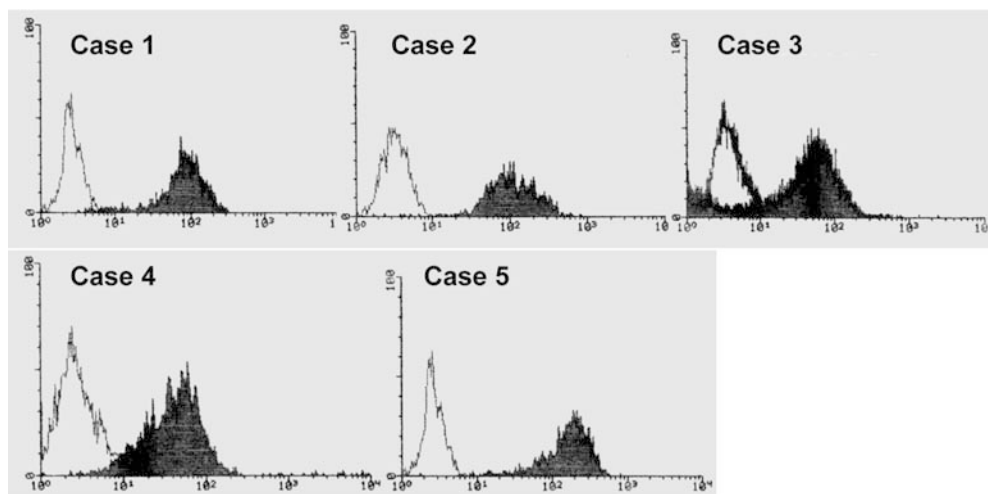
with tumor mRNA–pulsed DC. Results are presented as the mean values from at least three independently primed CD8$^+$ T-cell cultures from each patient. As shown in Fig. 2, cytotoxicity against autologous tumor cells was demonstrated for five patients at an effector to target ratio of 50, 25, 12.5, and 6.25 to 1. For these patients, tumor specific cytotoxicity was significantly inhibited by blocking MAb against HLA class I (significant at $P < 0.01$). Lysis of NK-sensitive K562 cells was also observed, notably for the CD8$^+$ T cells from these five patients. However, lysis of autologous tumor cells was significantly higher than lysis of K-562 cells ($P < 0.01$ for patients 1, 2, and 5). These results suggest that specific, HLA class I–restricted cytotoxicity against autologous tumor antigens is a major component of the CD8$^+$ T-cell response following stimulation with tumor mRNA–pulsed DC, although an NK-like CD8$^+$ cytotoxic T-cell response was also detected from these patients. In contrast to the cytotoxic responses generated from patients 1, 2, and 5, we were able to generate only a very low level of T-cell-mediated cytotoxicity from a single DC-primed CD8$^+$ T-cell culture from patient 3 and 4 (Fig. 2). For all five patients, minimal levels of cytotoxicity against autologous Con A–stimulated lymphoblasts (Fig. 2) or EBV-transformed LCL (data not shown) were observed. Also, for all five patients, minimal levels of cytotoxicity against autologous tumor cells with purified CD8$^+$ T cells 3 weeks after stimulation of T-cell cocultures with normal brain mRNA–pulsed DC were observed. Cytotoxicity against autologous tumor cells was demonstrated for five patients at an effector to target ratio of 50:1 as $8 \pm 5\%$.

Phenotypic analysis of CD8$^+$ T cells

Flow cytometric analysis was used to determine the phenotype of the populations of tumor-stimulated CD8$^+$ T cells derived from the five patients. All the cells were CD3/CD8$^+$ and CD4$^-$, with a variable proportion of CD56 antigen positive cells. CD8$^+$ T cells were also CD25$^+$ and HLA-DR+ (data not shown).

Cytokine production

In unstimulated cultures, IL-10 production was considerably increased in two cases (cases 3 and 4), compared with the other three cases (cases 1, 2, and 5). In OKT3-stimulated cultures, there was no significant difference in IL-10 levels. CD8$^+$ T cells obtained from cases 3 and 4

▶

Fig. 2 Tumor specific CD8$^+$ CTL responses induced by tumor mRNA–pulsed DCs in patients with glioma, measured in a 6 h ^{51}Cr-release assay. Percentage lysis ($\pm$ standard deviation) at effector to target ratios of 50, 25, 12.5, and 6.25 to 1 is shown. Anti-HLA class I blocking antibody (W6/32) was used at 50 μg/ml. Values are presented as the mean of three independently primed T-cell cultures. *$P < 0.01$
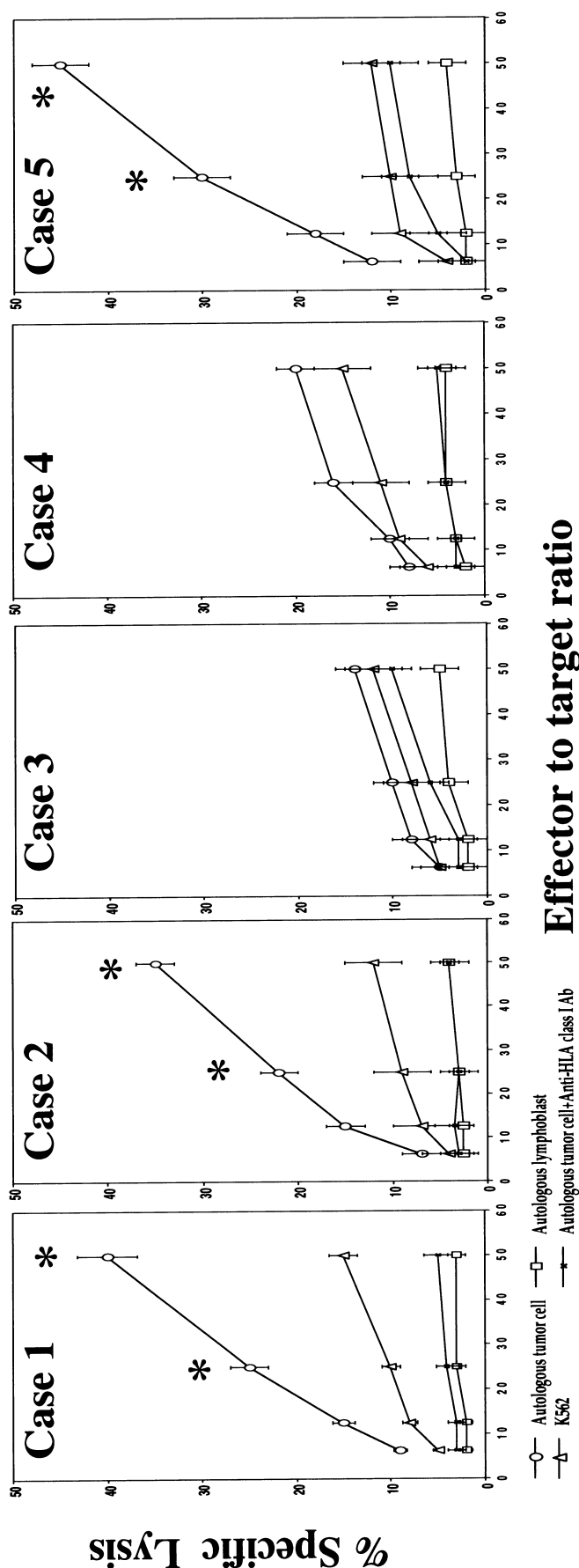
636

Table 2 IFN-γ and IL-10 production by tumor specific CD8$^+$ T cells. Results, given as means ($\pm$ standard deviation), are expressed in pg/ml OKT3, anti-CD3. (A) unstimulated cultures; (B) OKT3 stimulation

*$P < 0.01$

Cytokine	Case 1	Case 2	Case 3	Case 4	Case 5
(A)	**Basal**				
IFN-γ	8.5 ($\pm$2.0)	7.3 ($\pm$1.6)	1.6 ($\pm$1.0)*	1.8 ($\pm$1.2)*	10.0 ($\pm$3.2)
IL-10	12.3 ($\pm$4.2)	15.8 ($\pm$4.3)	55.6 ($\pm$10.0)*	45.5 ($\pm$10.2)*	10.6 ($\pm$3.8)
(B)	**OKT3**				
IFN-γ	550.2 ($\pm$85.2)	525.5 ($\pm$70.5)	425.0 ($\pm$65.2)	450.6 ($\pm$72.5)	573.5 ($\pm$86.9)
IL-10	840.5 ($\pm$120.5)	825.2 ($\pm$115.2)	915.0 ($\pm$140.5)	880.5 ($\pm$135.5)	865.5 ($\pm$130.8)

show a constitutively increase of IL-10 in vitro production compared with cases 1, 2, and 5 ($P < 0.01$). This significant difference is not maintained following OKT3 stimulation. Stimulation of CD8$^+$ T cells by OKT3 increased IL-10 production similarly in five patients (Table 2).

A basal decrease of IFN-γ production in patients 3 and 4, compared with patients 1, 2, and 5 ($P < 0.01$). The increase of IFN-γ levels after mitogenic stimulation led to similar levels among five patients, these could have been activated but in cases 3 and 4, the patients' T lymphocytes had a limited percentage of activation (Table 2).

Then in absence of mitogenic stimuli there was a definite activation in cases 3 and 4 of the patient's T lymphocytes toward T$_H$2 phenotype.

Discussion

To date, the possible immunization of cancer patients using defined tumor antigens has been limited to human tumor types in which candidates for tumor antigens have been identified. Despite the elucidation of a number of defined tumor antigens from human tumors, a recent study in patients with melanoma revealed that most of the T cells specific for the tumor did not recognize well-known antigens, suggesting that the tumor antigens may vary from patient to patient or are undefined [1]. In an attempt to overcome these major obstacles, several investigators have recently reported induction of effective tumor immune response using DCs pulsed with unfractionated tumor-derived materials, such as acid eluted peptides, tumor lysate, or messenger RNA (mRNA) [4, 13, 24]. DC vaccine treatment might be extended to patients with a variety tumors, provided that in vitro confirmation of tumor-specific CTL activity has been achieved.

In this study, we have shown that strong CD8$^+$ cytotoxic T-cell responses against autologous glioma cells can be elicited in patients with malignant glioma following stimulation of PBMCs with glioma mRNA–pulsed DC. Although CD8$^+$ cytotoxic T cells stimulated in this fashion showed some lytic activity against NK-sensitive K562 cells, much higher levels of cytotoxicity against autologous glioma cells were observed from three of five these patients. Furthermore, tumor-specific cytotoxicity was significantly inhibited by blocking MAb

specific for HLA class I, indicating that a HLA-restricted CD8$^+$ cytotoxic T-cell response against glioma antigens was a major component of the response. Autologous LCL or Con-A activated blasts were not killed by glioma specific CTLs, indicating that while these CTLs were highly cytolytic for autologous glioma cells, they failed to kill autologous normal cells. Also DC-pulsed normal brain mRNA failed to induce cytolytic activity against autologous glioma cells, suggesting the lack of autoimmune response. Unfractionated glioma RNA–transfected DCs elicit responses to a host of yet unidentified glioma antigens. Immunization with DC transfected with autologous glioma RNA may provide a strategy to induce glioma-specific T cells from patients against a broad repertoire of glioma antigens, without the need to know the antigenic profile of each patient.

T-cell-mediated protection against tumors is thought to be promoted by type 1 cytokine responses and impaired by type 2 cytokine responses [16]. In general, type 1 T cells express IL-2, IFN-γ, and TNF-β and favor cell-mediated immunity, delayed-type hypersensitivity, macrophage activation, and the production of opsonizing antibodies and are cytotoxic, whereas type 2 T cells express IL-4, IL-5, IL-6, IL-10, and IL-13, and favor humoral responses, providing for antibody production, and promote both mast cell growth and eosinophil differentiation and activation, resulting in humoral responses, and are noncytotoxic. Consistent with this notion, recent studies have showed significant dysfunction of type 1 T-cell responses in patients with cancer, suggesting that progression of disease may be associated with a preferential type 2 T-cell response [8, 16]. The reasons for the lack of a cytotoxic response against autologous glioma cells by glioma mRNA–pulsed DC stimulated CD8$^+$ T cells from these primings for patient 3 and 4, are open to discussion. We consider that a strong type 2 bias in the CD8$^+$ T-cell response accounts for the lack of cytotoxic effector function from these patients. Supporting this idea, CD8$^+$ T cells isolated during these ineffective primings secreted large amounts of IL-10 and smaller amounts of IFN-γ as detected by ELISA. There is a basal decrease of T$_H$1 cytokines and an increase of T$_H$2 cytokines. Extensive glioma progression may thus be associated with tumor specific tolerance.

Taken together, the findings of this study show that patients carrying locally advanced glioma can elicit a powerful cytotoxic T-cell response against their autolo-

gous glioma in vivo following glioma mRNA-loaded-DC stimulation. However, evidence of glioma specific tolerance in some patients may account for a significant barrier to DC-based immunotherapy. In this group of patients, attempts to overcome established T_H2 responses with the use of strong T_H1 agents such as IL-12 and IFN-γ might have therapeutic implications.

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